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1 **Running title:**

2 MATE-type citrate transporter in legume

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1 **Title:**

2 LjMATE1, a Citrate Transporter Responsible for Iron Supply to Nodule Infection Zone of *Lotus*  
3 *japonicus*.

4

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16

17 **Abbreviations:**

18 ARA, acetylene reduction activity; DAB, diaminobenzidine; dpi, days post inoculation; GUS,  $\beta$

19 -glucuronidase; ICP-MS, inductively coupled plasma mass spectrometry; MATE, multidrug and

20 toxic compound extrusion; MBS, modified Barth's saline; NAS, nicotianamine synthase;

21 pLjMATE1, *LjMATE1* promoter region; RNAi, RNA interference; SNF, symbiotic nitrogen

22 fixation.

1

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3 AB649311: The nucleotide sequence reported in this paper has been submitted to DDBJ under

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5

1    **Abstract:**

2    Symbiotic nitrogen fixation by intracellular rhizobia within legume root nodules requires the  
3    exchange of nutrients between host plant cells and their resident bacteria. While exchanged  
4    molecules imply nitrogen compounds, carbohydrate, and also various minerals, the knowledge  
5    on molecular basis of plant transporters that mediate those metabolite exchanges are still limited.  
6    In this study, we have shown that a multidrug and toxic compound extrusion (MATE) protein,  
7    LjMATE1, is specifically induced during nodule formation, which nearly paralleled the nodule  
8    maturation, in a model legume *Lotus japonicus*. Reporter gene experiments indicated that the  
9    expression of *LjMATE1* was restricted to the infection zone of nodules. To characterize the  
10   transport function of LjMATE1, we conducted biochemical analysis using heterologous  
11   expression system with *Xenopus* oocyte, and found that LjMATE1 is a specific transporter for  
12   citrate. Physiological roles of LjMATE1 was analyzed with RNA interference (RNAi) line,  
13   which revealed limited growth of RNAi line under nitrogen deficiency condition with  
14   inoculation of rhizobia compared to the control. It was noteworthy that, Fe localization was  
15   clearly altered in nodule tissues of RNAi line. These results strongly suggest that LjMATE1 is a  
16   nodule-specific transporter that assists the Fe translocation from the root to nodules by  
17   providing citrate.

18

19    **Key words:**

20    Citrate, Fe translocation, *Lotus japonicus*, MATE, nodulation,

21

1 **Introduction**

2 Nitrogen-fixing symbiosis between legume plants and soil bacteria called rhizobia is one of the  
3 most important symbioses on the earth. They are not only a major source of fixed nitrogen in  
4 natural ecosystems, but also the single largest natural source of nitrogen for agriculture (Smil  
5 1999). Symbiotic nitrogen fixation (SNF) in legumes takes place in specialized organs called  
6 nodules that develop from dedifferentiated root cells after infection by rhizobia in the soil in a  
7 species-specific manner. Via infection threads rhizobia colonize in developing nodule tissue  
8 (Brewin 1991) and ultimately they enter cortical cells by endocytosis (Verma and Hong 1996).  
9 This nodule development is accompanied by coordinated differentiation of both plant and  
10 bacterial cells, which results in forming both infected and uninfected plant cells, which are  
11 developed in a mosaic form. During nodulation, global change in gene expression was involved  
12 in both partners (Colebatch et al. 2004, Kouchi et al. 2004, Uchiumi et al. 2004). Many of these  
13 changes strongly affect both plant and bacterial metabolisms and transports, which become  
14 specialized for supporting the exchange of reduced carbon and other nutrients from the plant to  
15 bacteroids, and fixed nitrogen from the bacteroids to the plant (White et al. 2007). It is  
16 postulated that many transporters are involved in the exchange of these nutrients, and thus for  
17 several transporters have been identified, e.g. SST1, a symbiotic sulfate transporter in *Lotus*  
18 *japonicus* (Krusell et al. 2005) and an allantoin transporter, PvUPS1 in french bean (*Phaseolus*  
19 *vulgaris*) (Pelissier et al. 2004), but overall understandings of membrane transport systems in  
20 nodule are still very limited.

21 Multidrug and toxic compound extrusion (MATE) proteins widely occur in bacteria, fungi,  
22 mammals, and also in plants (Omote et al. 2006). Apparently plants have a higher diversity of

1 MATE-type transporters than bacteria and animals e.g., there are 56 MATE orthologs in  
2 *Arabidopsis thaliana*, while only two members are found in human genome. AtDTX1 was the  
3 first identified plant MATE, which was isolated from the Arabidopsis cDNA library using a  
4 bacterial mutant defective in multidrug resistance (Li et al. 2002). AtDTX1 showed efflux  
5 activity for a variety of xenobiotics, however, further studies on other plant MATEs revealed  
6 that many of them showed rather restricted substrate specificity for their own metabolites such  
7 as flavonoids, alkaloids, and citrate. In particular, MATE-type transporters, which transport  
8 citrate, are reportedly involved in iron (Fe) translocation or aluminum (Al) detoxification in  
9 plants. For instance, FRD3 from Arabidopsis demonstrated as a citrate transporter is localized at  
10 the pericycle and cells internal to the pericycle cells in roots, and is required for Fe translocation  
11 from the roots to the shoots (Green and Rogers 2004, Durrett et al. 2007). Defects in this  
12 transporter resulted in the precipitation of Fe in the root vasculature (Green and Rogers 2004,  
13 Durrett et al. 2007). Also in monocots, a rice MATE transporter OsFRDL1, transports citrate at  
14 the pericycle cells of the roots, and was suggested to be involved in Fe translocation in the  
15 xylem as well (Yokosho et al. 2009). On the other hand, two studies have shown in 2007 that  
16 Al-induced secretion of citrate in barley (*Hordeum vulgare*) and sorghum (*Sorghum bicolor*) is  
17 mediated through MATE transporters (Furukawa et al. 2007, Magalhaes et al. 2007). Secretion  
18 of citrate from the roots is a mechanism of Al resistance in many plant species (Ma et al. 2001,  
19 Kochian et al. 2005, Delhaize et al. 2007). In addition, more recent studies have shown that two  
20 soybean MATE-type transporters, GmFRD3a and GmFRD3b, as well as ScFRDL1 from *Secale*  
21 *cereal* mediated Fe translocation (Rogers et al. 2009, Yokosho et al. 2010).

22 In order to comprehend the membrane transporters involved in SNF we have performed

1 transcriptome analysis following laser microdissection in a model legume *Lotus japonicus*.  
2 From this microarray experiments, a MATE-type transporter (*LjMATE1*) was identified as a  
3 nodule-specific gene in *L. japonicus* (Takanashi et al. 2012b). In this study, we demonstrate that  
4 *LjMATE1* can transport citrate, and assist the translocation of Fe to nodule infection zone,  
5 which is essential to support nodule function.

6

## 7 **Results**

### 8 **Expression analysis of *LjMATE1* during nodulation**

9 Based on our microarray analysis in nodules of *L. japonicas*, a MATE-type transporter gene  
10 (*LjMATE1*) is suggested to be involved in nitrogen fixation in this plant due to the high  
11 expression in the infection zone. To confirm the *in silico* data experimentally, expression  
12 analysis was carried out. Using gene-specific primers we found that the expression of *LjMATE1*  
13 was specifically observed in nodules of *L. japonicus* (Fig. 1A). Time-course experiment at five  
14 different nodulation stages revealed that the gene expression of *LjMATE1* was dramatically  
15 increased at 7 days post inoculation (dpi) of *Mesorhizobium loti* and kept high level during SNF  
16 (Fig. 1B).

17 To analyze the tissue specific expression of *LjMATE1* in nodules in more detail, we isolated  
18 1.7 kb fragment upstream of the translational initiation codon from *L. japonicus* genomic DNA,  
19 which contains the putative *LjMATE1* promoter region (*pLjMATE1*), and was fused to  $\beta$   
20 -glucuronidase (GUS) reporter gene. The binary vector containing *pLjMATE1::GUS* reporter  
21 system was then introduced into *L. japonicus* with hairy root transformation method. GUS  
22 expression analysis clearly showed that *LjMATE1* was expressed only in nodules and no



1 expression was seen in root tissues through nodulation (Fig. 1C-H). To identify the cell type  
2 where *LjMATE1* is expressed, nodule sections were prepared after GUS staining. Microscopic  
3 observation revealed that the GUS staining was observed mainly in the infected cells of nodules  
4 at 7 and 21 dpi (Fig. 1D, F). While, GUS staining decreased in central infection zone at 35 dpi  
5 (Fig. 1H).

6

### 7 **LjMATE1 has transport activity for citrate**

8 Plant MATE proteins can be divided into two distinct groups by their amino acid sequences,  
9 which seems to reflect their transport substrate, i.e., citrate and others. LjMATE1 belongs to a  
10 clade, in which all reported citrate-transporting MATE proteins are clustered, with high amino  
11 acid sequence similarity with GmFRD3a (72%) and GmFRD3b (71%), which are involved in  
12 Fe translocation from root to aerial parts (Rogers et al. 2009) (Fig. 2). To evaluate if LjMATE1  
13 has the activity to transport citrate as a substrate, we expressed LjMATE1 in *Xenopus* oocytes.  
14 Fig. 3 showed the transport assay using radioisotope-labeled citrate. The data indicated that the  
15 efflux activity for citrate was significantly higher in the oocytes pre-injected with LjMATE1  
16 cRNA than in oocytes injected with water as a negative control. This efflux activity of  
17 LjMATE1 was almost the same with HvAACT1, a previously reported citrate-transporting  
18 MATE (Supplementary Fig. S1) (Furukawa et al. 2007). We also examined transport activity of  
19 malate, which is used as energy source in bacteroids (Price et al. 1987), however, oocytes  
20 pre-injected with LjMATE1 cRNA did not show specific transport activity for malate compared  
21 with control (Fig. 3).

22

1 **Growth phenotype of *LjMATE1* knockdown line**

2 To determine the physiological role of *LjMATE1*, we suppressed its expression in transgenic  
3 plants by RNA interference (RNAi). Among 20 stable transformants, appreciable suppression of  
4 *LjMATE1* expression was found in an RNAi line (*ljmate1*) at T2 generation (Fig. 4A). As  
5 control, we used other RNAi line (*LjMATE1*) in which *LjMATE1* gene expression was not  
6 suppressed in addition to wild-type. The T2 RNAi line grew normally with nitrogen nutrient  
7 supplementation, and no apparent phenotype difference was observed compared to the wild-type.  
8 While when plants were inoculated with *M. loti* under nitrogen deficiency condition, the RNAi  
9 plant showed clear growth defect phenotype (Fig. 4B, C), suggesting that the ability of SNF was  
10 reduced in the knockdown line. Then we observed nodule phenotype of the *ljmate1* line.  
11 Accompanied with normal pink nodules that were normally seen in control plants, about one  
12 third of nodules in *ljmate1* revealed greenish appearance (Fig. 5A-D). The nodule size of  
13 *ljmate1* was significantly smaller than that of control plants (Fig. 5E), and the acetylene  
14 reduction activity (ARA) of nitrogenase was also significantly reduced in *ljmate1* as well as the  
15 expression level of *leghemoglobin* (Fig. 5F, G).

16  
17 **Fe accumulation in *LjMATE1* knockdown nodules**

18 MATE-type transporters, which have transport activity for citrate, are known to be involved in  
19 either Fe translocation or Al resistance. To investigate whether *LjMATE1* mediates Fe  
20 translocation or not, Fe localization in nodules of RNAi plants was examined with Perls staining  
21 (Green and Rogers 2004). While visible Fe precipitation was not observed in control nodules  
22 (Fig. 6A, B), strong Perls staining was detected at root-nodule junction area and nodule vascular

1 bundle of RNAi nodules (Fig. 6C, D), indicating altered Fe localization in *ljmate1* nodules. This  
2 staining pattern was not observed in *LjMATE1* nodules (control) (Fig. 6E, F). To observe Fe  
3 localization in more detail with nodule sections, the iron staining was enhanced by second  
4 reaction with diaminobenzidine (DAB) and hydrogen peroxide (Roschztardt et al. 2009). Fig.  
5 7A and 7B clearly showed that Fe was accumulated mainly in the infection zone of wild-type  
6 nodule, especially in infected cells, where a large amount of leghemoglobin is accumulated. It is  
7 to be noted that almost no Fe accumulation was observed in root vascular bundle of wild-type  
8 plant (Fig. 7C). In contrast, Fe localization in nodules of *ljmate1* was seen in infected cells, but  
9 the level was much low in particular in the central zone (Fig. 7D, E). Another clear difference  
10 was observed in the root vascular bundle of *ljmate1*, where high Fe deposition was detected (Fig.  
11 7F). Fe accumulation pattern in the nodules of another control *LjMATE1* was almost the same  
12 with wild-type nodules (Fig. 7G-I).

13 For quantitative comparison, we determined Fe content in the nodules using inductively  
14 coupled plasma mass spectrometry (ICP-MS). The Fe content in the nodules of stable *ljmate1*  
15 was significantly lower compared to that in wild-type (decreased by 43.2%) (Fig. 8), and the  
16 contents of other metals, such as Zn and Mg, were not significantly changed between wild-type  
17 and *ljmate1* (Fig. 8). These results strongly suggested that *LjMATE1* is involved in Fe  
18 translocation in nodules by releasing citrate, which is essential to support the function of SNF.

19

## 20 **Discussion**

21 Fe storage is of a special importance in nodules, especially in infected cells, because of a high  
22 accumulation of leghemoglobin and nitrogenase, both of which requires Fe for their activity.

1 Several studies demonstrated a part of mechanisms of Fe accumulation in nodules. For instance,  
2 Fe (II) transport activity across peribacteroid membrane was measured in soybean nodules  
3 (Moreau et al. 1998), and later a transporter that mediates the Fe movement at peribacteroid was  
4 identified as GmDMT1 (Kaiser et al. 2003). Fe (III) is also provided across peribacteroid  
5 membrane when chelated with several organic acids such as citrate, and accumulates in the  
6 peribacteroid space, where Fe (III) reductase activity is present (LeVier et al. 1996). In contrast  
7 with Fe transport across peribacteroid membrane, the mechanisms of Fe translocation from root  
8 tissues to nodules, especially to infected cells, have been unknown. In this study, we  
9 characterized LjMATE1 and found that this MATE-type protein mediates Fe translocation into  
10 infected cells by releasing citrate into apoplast in infection zone.

11 Expression analysis by qPCR showed that *LjMATE1* expression was induced at 7 dpi and  
12 reached a maximum at 12 dpi (Fig. 1B). This induction pattern is similar to the expression of  
13 leghemoglobin and nitrogenase activity. Moreover, promoter analysis revealed that expression  
14 of *LjMATE1* was restricted to the infection zone of nodules, suggesting LjMATE1 is directly  
15 involved in SNF.

16 The physiological role of LjMATE1 was demonstrated by generating transgenic plants, in  
17 which the expression of *LjMATE1* was suppressed by RNAi. Previous reports on Fe deficient  
18 mutants in *Arabidopsis frd3* and rice *osfrd11* showed chlorosis in cotyledons or leaves especially  
19 under low Fe condition (Durrett et al. 2007, Yokosho et al. 2009). However, the knockdown  
20 line *ljmate1* demonstrated in this study did not show such phenotype in nitrogen sufficient  
21 condition, as LjMATE1 functions exclusively in nodules as indicated in the expression analysis  
22 (Fig. 1A). When *ljmate1* plants were inoculated with rhizobia under nitrogen deficiency

1 condition, the growth was apparently suppressed compared to wild-type, but not with  
2 supplementation of nitrogen nutrient (Fig. 4C). In addition, *ljmate1* have many greenish nodules  
3 (Fig. 5B, D), which are known as inactive in SNF. Indeed, ARA of *ljmate1* nodules was  
4 significantly reduced (Fig. 5F), indicating that LjMATE1 is required for SNF and suppression of  
5 *LjMATE1* caused fix- nodules.

6 DAB-enhanced Perls staining was first applied in plant tissue to detect Fe accumulation in  
7 Arabidopsis embryos, in which the staining was too weak by the single Perls staining alone  
8 (Roschztardt et al. 2009). In this study we have employed this DAB-enhancement to monitor  
9 the Fe accumulation in nodule sections. In wild-type nodules, Fe accumulation was detected  
10 mainly in the infection zone, which is consistent with the physiological functions of Fe in  
11 nodules (Fig. 7A, B). However, Fe staining was apparently fainter in the infection zone of  
12 *ljmate1* nodules particularly in the central region (Fig. 7D, E). Then, we determined Fe contents  
13 in nodules using ICP-MS, and found the reduced Fe concentration in *ljmate1* nodules (Fig. 8).  
14 These results strongly suggest the involvement of LjMATE1 in Fe accumulation in nodules by  
15 increasing Fe mobility.

16 Moreover, unusual Fe deposition was seen in the root vascular bundles in the *ljmate1* plant  
17 as well (Fig. 6D, 7F), which may be a secondary phenomenon of stagnant Fe delivery into the  
18 nodules, as the expression of *LjMATE1* is restricted to the infection zone of nodules (Fig. 1C-H).  
19 The spacial difference in tissues between the relevant gene expression and the Fe accumulation  
20 was also observed in rice (Kobayashi et al. 2010), i.e., rice OsYSL2 functioning as a transporter  
21 of Fe-nicotianamine complex is expressed mainly in the phloem cells of leaves and leaf sheaths,  
22 whereas the RNAi lines of OsYSL2 showed increased Fe accumulation in roots (Koike et al.

1 2004, Ishimaru et al. 2010).

2 Plants utilize highly sophisticated mechanisms for Fe uptake and translocation among  
3 organs. As naturally abundant Fe<sup>3+</sup> is hardly water-soluble, plants secrete various molecules to  
4 increase the Fe solubility, for instance, nicotianamine and citrate to form Fe-chelator complex  
5 (Conte and Walker 2011). The accumulation of nicotianamine in nodules has not been reported,  
6 however, Hakoyama and co-workers showed that a nicotianamine synthase (NAS) that mediated  
7 the reaction from *S*-adenosylmethionine to nicotianamine, was expressed in a late stage of  
8 nodulation in *L. japonicus*, and they postulated that LjNAS2 is involved in Fe reuse from  
9 inactive nodule to other organs (Hakoyama et al. 2009). It is reported that citrate, which also  
10 serves as chelator of Fe, accumulates in nodules, while the citrate content decreases to  
11 approximately 60% when plant is inoculated by ineffective *Rhizobium* (Rosendahl et al. 1990).  
12 In this study, we could not demonstrate the transport direction of citrate by LjMATE1, but we  
13 presume that LjMATE1 effluxes citrate at plasma membrane into apoplast by the following  
14 reasons. 1) Fe accumulation is decreased in the infected cells when *LjMATE1* expression was  
15 suppressed (Fig. 7 and 8); 2) Fe movement in peribacteroid space is possible without citrate,  
16 where Fe (III) -chelator complex can be reduced to Fe (II) and/or bound with siderophores  
17 released by the bacteroid (LeVier et al. 1996, Wittenberg et al. 1996). For the uptake of  
18 Fe-citrate complex into infected cells other metal transporters may be involved. In fact, metal  
19 transporter genes, such as Zrt/Irt-like protein (ZIP) and natural resistance-associated  
20 macrophage protein (Nramp), are up-regulated during nodulation in our transcriptomic analysis  
21 besides *LjMATE1* (Takanashi et al. 2012b). Detailed characterization of these transporters will  
22 enable us to grasp the overall scheme of Fe exchange between bacteroids and plant cells.

1

## 2 **Material and Methods**

### 3 **Plant materials and growth conditions**

4 *L. japonicus* MG-20 Miyakojima was used in this work. Seeds were surface-sterilized with a  
5 1% sodium hypochloride solution for 10 min, rinsed five times with sterile distilled water, then  
6 germinated on water agar plate (0.8%). Five-day-old seedlings were transferred on sterile  
7 vermiculite with liquid 1/2 B&D medium (Broughton and Dilworth 1971) in plant box and  
8 grown in a cultivation chamber under a 16-h day/8-h night cycle at temperatures of 23°C.

9

### 10 **Expression analysis with rhizobia**

11 To obtain RNA samples, 9-day-old seedlings were inoculated with *M. loti* MAFF303099, which  
12 was cultured overnight in TY medium at 28°C. For organ-specific expression analysis, plants at  
13 19 dpi were sampled. Time course analysis was conducted by collecting aerial parts and  
14 underground parts respectively, at 0, 2, 4, 7, 12, and 19 dpi. Reverse transcription was carried  
15 out using a SuperscriptIII Reverse Transcriptase (Invitrogen), followed by semi-qPCR (95°C for  
16 1 min, 32 cycles at 95°C for 20 s, 55°C for 30 s and 72°C for 20 s) using Go Taq DNA  
17 Polymerase (Promega) with the sets of primers specific to *LjMATE1* (forward primer,  
18 5'-GCTACACAACCCATCAATGC-3' and reverse primer,  
19 5'-TGCAAATGAGACCATCACCA-3'). *Ubiquitin* (forward primer, 5'-  
20 ATGCAGATCTTCGTCAAGACCTTGAC-3' and reverse primer, 5'-  
21 ACCTCCCCTCAGACGAAGGA-3') was used as an internal control. For qPCR (95°C for 15  
22 min, 40 cycles at 94°C for 10 s, 56°C for 20 s and 72°C for 25 s), DyNAMO HS SYBR Green

1 qPCR Kit (Finnzymes) was used. Real-time detection of PCR products was performed using  
2 Roter-Gene 3000A (Corbett Research).

3

#### 4 **Histochemical analysis of promoter-GUS transformant of *L. japonicus***

5 A fragment of 1.7 kb upstream the start codon of *LjMATE1* was amplified from genome DNA  
6 of MG-20 using Phusion High-Fidelity DNA Polymerase (Finnzymes) with a set of specific  
7 primers, forward primer,  
8 5'-ACAAGTTTGTACAAAAAAGCAGGCTCTTGGAAGGGGCTGTCTTTT-3'; reverse  
9 primer, 5'-ACCACTTTGTACAAGAAAGCTGGGTGATATCCTAAATCTTATGTAA-3' (the  
10 underlined positions are non-native sequences of the *attB* recombination sites). The PCR  
11 product was subcloned into pDONR/Zeo (Invitrogen), and then transferred into a  
12 Gateway-compatible binary vector, pGWB3 (Nakagawa et al. 2007). The construct was then  
13 introduced into *L. japonicus* using hairy-root transformation mediated by *Agrobacterium*  
14 *rhizogenes* LBA1334, as previously reported (Kumagai and Kouchi 2003). The transformed  
15 plants were inoculated with *M. loti* and grown in a cultivation chamber under the condition  
16 mentioned above. Nodules were sampled at 7, 21, and 35 dpi, respectively, and GUS staining  
17 was performed as described previously (Takanashi et al. 2011)

18

#### 19 **Transport assay in *Xenopus* oocytes**

20 The full-length cDNA of *LjMATE* was cloned into the oocyte vector pXβG-evl. The plasmid  
21 was linearized with *SacII*, and cRNA was transcribed in vitro with T3 RNA polymerase  
22 (mMESSAGE mMACHINE kit; Ambion). Oocytes were isolated from adult female *Xenopus*



1 *laevis* frogs as described before (Ma et al. 2006). Selected oocytes were incubated for 1 day in  
2 modified Barth's saline (MBS) at 18°C until the injection of cRNA. A 50 nl of water solution  
3 containing 50 ng cRNA or not was injected into each oocyte with a nano-jector II (Drummond  
4 Scientific Company), followed by incubating in MBS at 18°C for 2 days. For determining  
5 citrate efflux activity, a 50 nl of 2.4 mM <sup>14</sup>C-labeled citrate or malate (2.3 nCi/oocyte) was  
6 injected. The oocytes were then washed four times with MBS buffer (pH 7.6) and then  
7 transferred into a 500 µl of fresh buffer at 18°C. After 30 min incubation, the external buffer  
8 was sampled and the oocytes were homogenized with 0.1N HNO<sub>3</sub>. The radioactivity of the  
9 buffer solution and homogenized oocytes was measured with a liquid scintillation counter  
10 (LIQUID SCINTILLATION SYSTEM; Aloka).

11

#### 12 **LjMATE1 RNAi transformants of *L. japonicus***

13 For *LjMATE1* suppression by RNA interference, a 245 bp fragment (position 226-471) was  
14 amplified from the *LjMATE1* cDNA using a set of primers containing *attB* recombination sites  
15 for Gateway vector, forward primer, 5'-  
16 ACAAGTTTGTACAAAAAAGCAGGCTGGAGTTGCCATTGCTTTGTT -3'; reverse primer,  
17 5'- ACCACTTTGTACAAGAAAGCTGGGTCTCAACACTTGCAGCCAAAA -3' (the  
18 underlines indicate specific sequences of the *attB* recombination sites). The PCR product was  
19 subcloned into pDONR/Zeo (Invitrogen), and then transferred into a Gateway-compatible  
20 binary vector, pUB-GWS-Hyg (Maekawa et al. 2008). The construct was used for plant  
21 transformation as described previously (Thykjaer et al. 1997). For phenotypic analysis,  
22 wild-type and T2 generation were grown either with supplementation of nitrogen nutrient

1 without rhizobia inoculation, or under nitrogen deficiency condition with rhizobia inoculation.  
2 Plants were sampled and photographed at 28 dpi. The nitrogenase activity was measured by the  
3 acetylene reduction assay (Maruya and Saeki 2010). For expression analysis of leghemoglobin,  
4 the set of primers specific to *leghemoglobin* was used (forward primer,  
5 5'-TTTGAGCACTGCTTGGGGAGTAGCT-3' and reverse primer,  
6 5'-AGGCATGCAAAACCAGAAAC-3'). The qPCR condition was described above. For hairy  
7 root transformation the RNAi fragment in pDONR/Zeo was transferred into a binary vector,  
8 pUB-GWS-GFP (Maekawa et al. 2008). The transformed hairy roots, selected with GFP signal,  
9 were inoculated with *M. loti* and sampled at 28 dpi.

10

### 11 **Perls staining**

12 For Perls staining, pink nodules were vacuum-infiltrated with equal volumes of 4% (v/v) HCl  
13 and 4% (w/v) K-ferrocyanide for 15 min, and incubated for 30 min at room temperature  
14 (Roschztardt et al. 2009). For the signal intensification with DAB and H<sub>2</sub>O<sub>2</sub>, the Perls stained  
15 nodules were fixed and embedded in Technovit 7100 as previously reported (Takanashi et al.  
16 2012a). The sections (15 μm) were placed on glass slides, and then the intensification procedure  
17 was applied as described by Meguro et al. (Meguro et al. 2007).

18

### 19 **Measurement of the Fe content in nodules**

20 Seeds were germinated on a water agar plate. Five-day-old seedlings were transferred to sterile  
21 vermiculite with liquid 1/2 B&D medium in a plant box. After 2 days plants were inoculated by  
22 bacteria. At 28 dpi, the pink nodules were collected and dried in an oven at 70°C for 2 days.

1 Samples were then subjected to digestion with 0.5 ml of 61 % HNO<sub>3</sub> in a 2 ml plastic tube. The  
2 samples were heated up to 125°C for 5 hours and then diluted with 5% HNO<sub>3</sub>. The metal  
3 concentration was determined by ICP-MS (7700X; Agilent Technologies).

4

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9

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14 LBA1334.

15

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20  
21

1 **Figure legends**

2 **Fig. 1** Expression analyses of *LjMATE1*. (A) Tissue-specific semi-quantitative RT-PCR analysis  
3 of *LjMATE1*. (B) qPCR of *LjMATE1* in underground parts at different development stages of  
4 nodulation. Expression of *LjMATE1* was detected only in nodule, which increased at 7 dpi and  
5 kept high level of expression during nodulation. Values represent mean  $\pm$  SD (n = 3). (C-H)  
6 GUS staining of *pLjMATE1::GUS* transformant. Whole nodules (C, E, G) and cross sections (D,  
7 F, H) of nodule at 7 (C, D), 21 (E, F), and 35 (G, H) dpi, respectively. Stained infected cells  
8 were found at 7 and 21 dpi (D, F). GUS expression mostly disappeared at 35 dpi (H). Bars =  
9 200  $\mu$ m.

10

11 **Fig. 2** An unrooted phylogenetic analysis of plant MATE transporters. Phylogenetic tree was  
12 generated using MEGA 5.0 software (Tamura et al. 2011). *LjMATE1* and reported plant MATE  
13 transporter sequences were aligned using the ClustalW program. The neighbor-joining method  
14 was then used to construct a phylogenetic tree with 1,000 bootstrap replicates. MATE proteins  
15 in red were reported to be involved in Fe translocation or Al detoxification. Accession numbers  
16 and AGI codes are: AM1, FJ264202; AM3, FJ264203 (*Vitis vinifera*); AtALF5, At3g23560;  
17 AtDTX1, At2g04070; AtEDS5, At4g39030; AtFRD3, At3g08040; AtMATE, At1g51340;  
18 AtTT12, At3g59030; AtZRIZI, At1g58340 (*Arabidopsis thaliana*); GmFRD3a, EU591739;  
19 GmFRD3b, EU591741 (*Glycine max*); HvAACT1, AB302223 (*Hordeum vulgare*); LaMATE,  
20 AY631874 (*Lupinus albus*); MtMATE1, FJ858726; MtMATE2, HM856605 (*Medicago*  
21 *truncatula*); MTP77, Q6V7U8 (*Solanum lycopersicum*); NtJAT1, AM991692; NtMATE1,  
22 AB286961 (*Nicotiana tabacum*); OsFRDL1, Q10PY7 (*Oryza sativa*); SbMATE, EF611342

1 (*Sorghum bicolor*); ScFRDL1, AB571881; ScFRDL2, AB571882 (*Secale cereale*); ZmMATE1,  
2 FJ015155 (*Zea mays*).

3  
4 **Fig. 3** Transport activity of LjMATE1 measured in *Xenopus* oocytes. The LjMATE1-expressing  
5 oocytes were injected with <sup>14</sup>C-labeled citrate or <sup>14</sup>C-malate and their release from the oocytes  
6 was determined 30 min after injection. LjMATE1 showed an efflux activity for citrate, but not  
7 for malate. Values represent mean ± SD (n = 4). \**p*<0.01 compared with control (H<sub>2</sub>O) by  
8 Student's t-test.

9  
10 **Fig. 4** Phenotype of 5-week-old *LjMATE1* RNAi knock-down lines. Plants were grown in  
11 vermiculite with (+N) or without (-N) nitrogen supplementation. (A) Expression analysis of  
12 *LjMATE1* in RNAi lines with qPCR. Values represent mean ± SD (n = 3). (B) Shoot length of  
13 control and RNAi lines under nitrogen deficiency condition. Values represent mean ± SD (n =  
14 16). (C) Growth phenotype of 5-week-old rhizobia-inoculated plant (28 dpi). Growth of *ljmate1*  
15 was clearly suppressed under nitrogen deficiency condition. Scale bar = 2 cm. (A, B) \**p*<0.01  
16 compared with controls by Tukey-Kramer test.

17  
18 **Fig. 5** Phenotype of *L. japonicus* nodules at 28 dpi. Nodule phenotype (A, WT; B, *ljmate1*; C,  
19 *LjMATE1*), number per plant (D, n = 10-18), size (E, n = 19-31), and ARA (F, n = 32-55) of  
20 wild-type and RNAi lines. (G) Expression level of leghemoglobin in nodules (n = 3). In *ljmate1*  
21 greenish nodules were observed as well as pink nodules, and reduced nitrogen fixation activity  
22 was measured. Scale bars = 1 mm. Values represent mean ± SD. \**p*<0.05, \*\**p*<0.01 compared

1 to controls by Tukey-Kramer test.

2

3 **Fig. 6** Fe accumulation in nodules was examined with Perls staining. Fe precipitation was not  
4 observed inside wild-type nodules from either top (A) or nodule-root junction (B) side. In  
5 *ljmate1* Fe accumulation was not seen from the nodule top side (C), while strong blue staining  
6 was detected at nodule-root junction (D, pointed by arrowhead) and nodule vascular bundle. In  
7 *LjMATE1* the blue staining was not observed at both top (E) and nodule-root junction (F) side.  
8 Scale bars = 200  $\mu$ m.

9

10 **Fig. 7** Typical Fe localization inside the nodules of wild-type (A-C), *ljmate1* (D-F) and  
11 *LjMATE1* (G-I). Perls staining was enhanced by DAB and H<sub>2</sub>O<sub>2</sub>. (A, B) High Fe accumulation  
12 was observed in infected cells in wild-type nodule. (C) Root vascular bundle was not stained in  
13 wild-type. (D, E) Fe accumulation in infection zone was reduced in *ljmate1*, especially in the  
14 central region. (F) Abnormality in the Fe accumulation was also detected in root vascular  
15 bundle. (G-I) Same Fe accumulation pattern was observed in *LjMATE1* nodule with wild-type.  
16 (A, D, G) Root vascular bundle was pointed by arrowheads. Scale bars = 100  $\mu$ m.

17

18 **Fig. 8** Fe and other metal contents in *L. japonicus* nodules determined by ICP-MS. The nodules  
19 of wild-type and *ljmate1* plants were sampled at 28 dpi. The Fe concentration of *ljmate1* was  
20 reduced to 56.8% of WT. Values represent mean  $\pm$  SD (n = 3). \**p*<0.05 compared with WT by  
21 Student's t-test.

Fig. 1

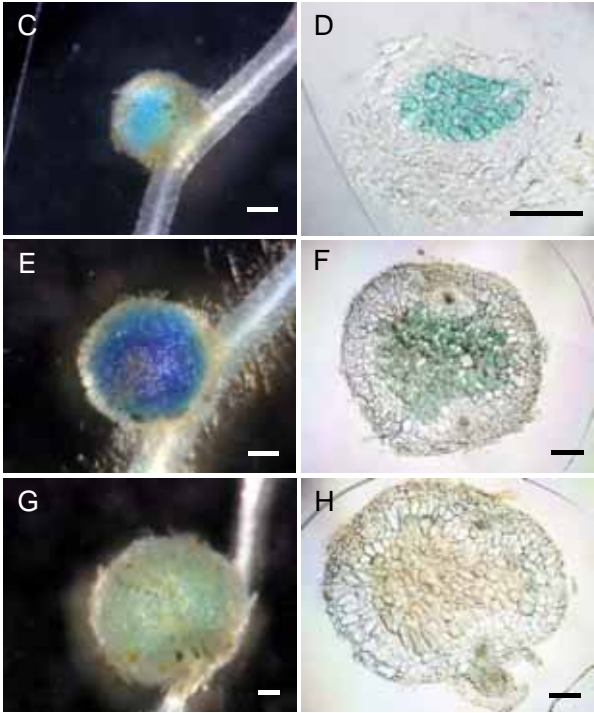
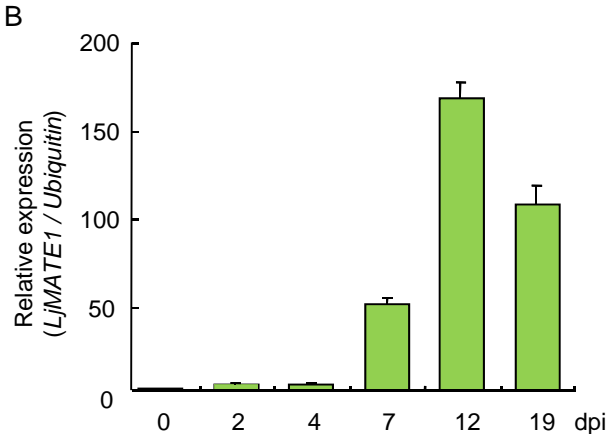
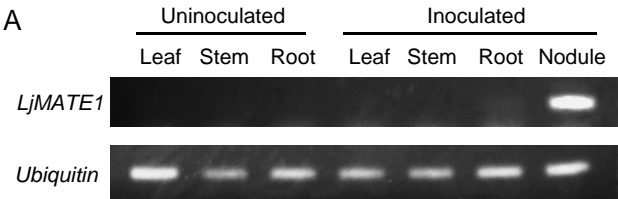


Fig. 2

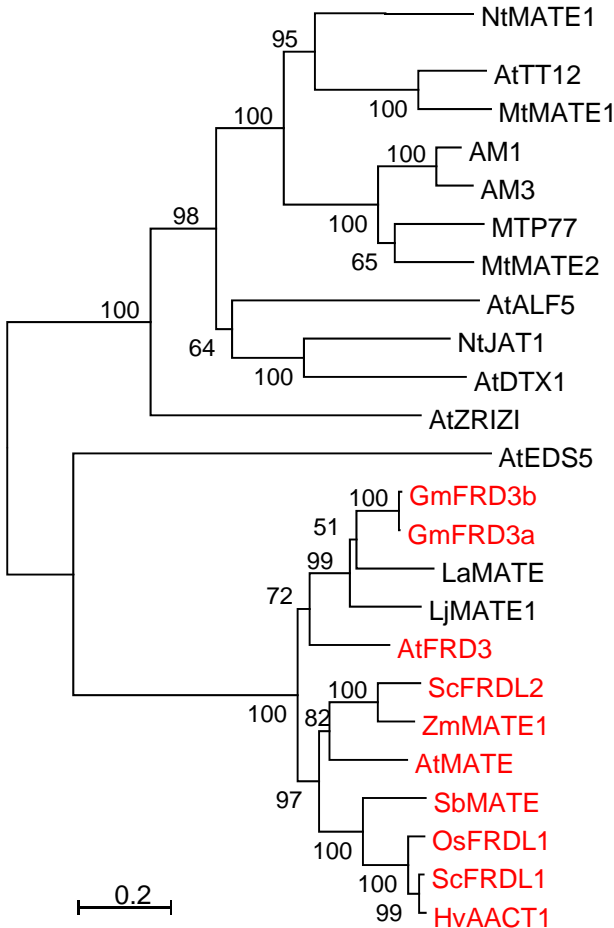


Fig. 3

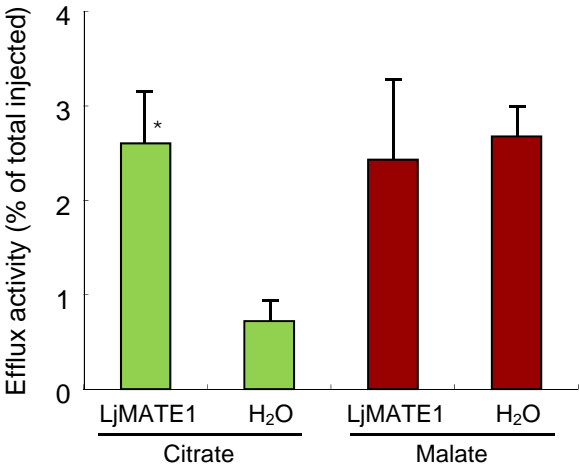




Fig. 4

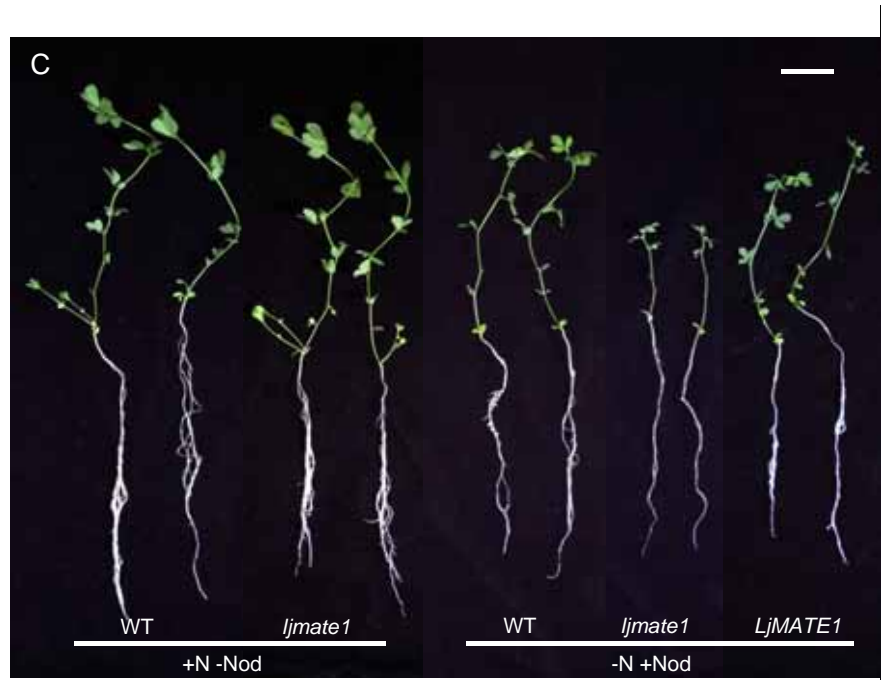
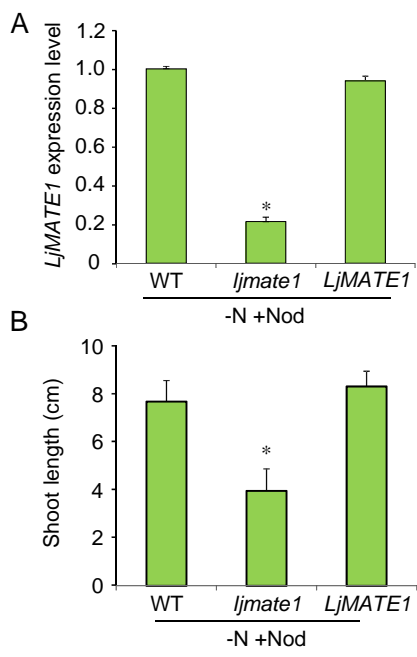


Fig. 5

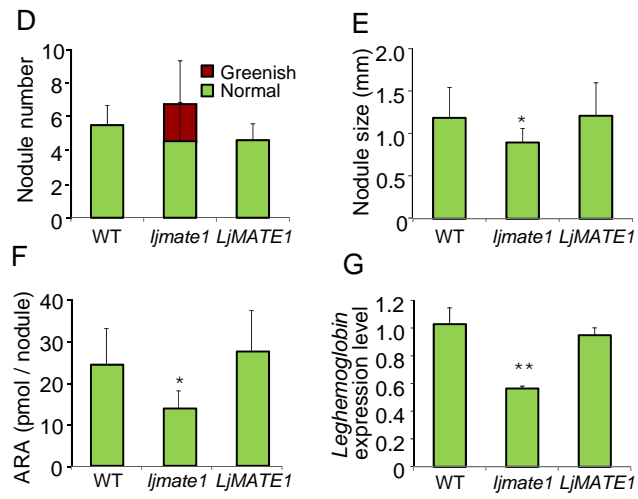
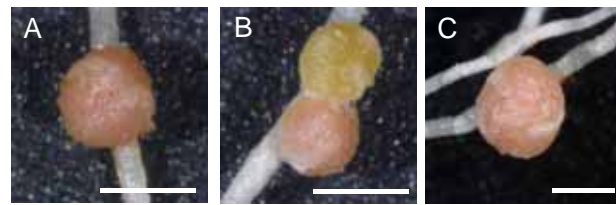


Fig. 6

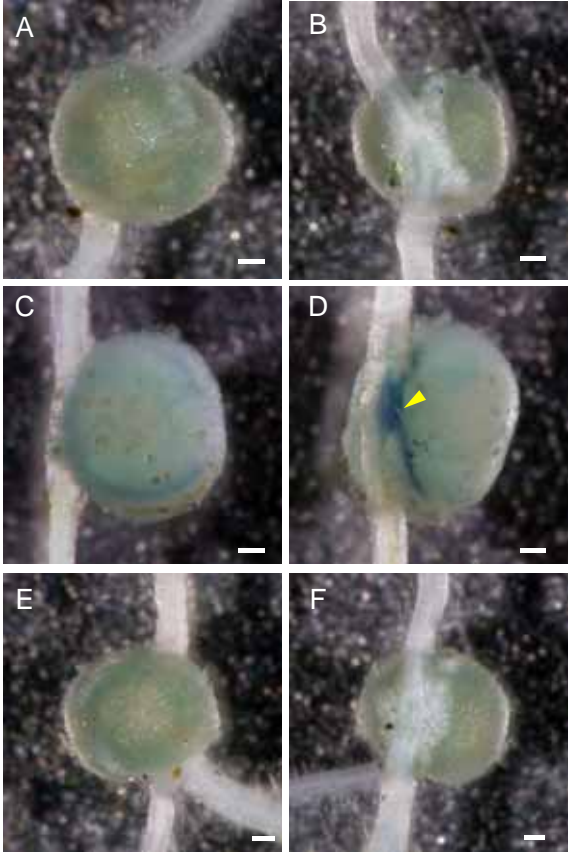


Fig. 7

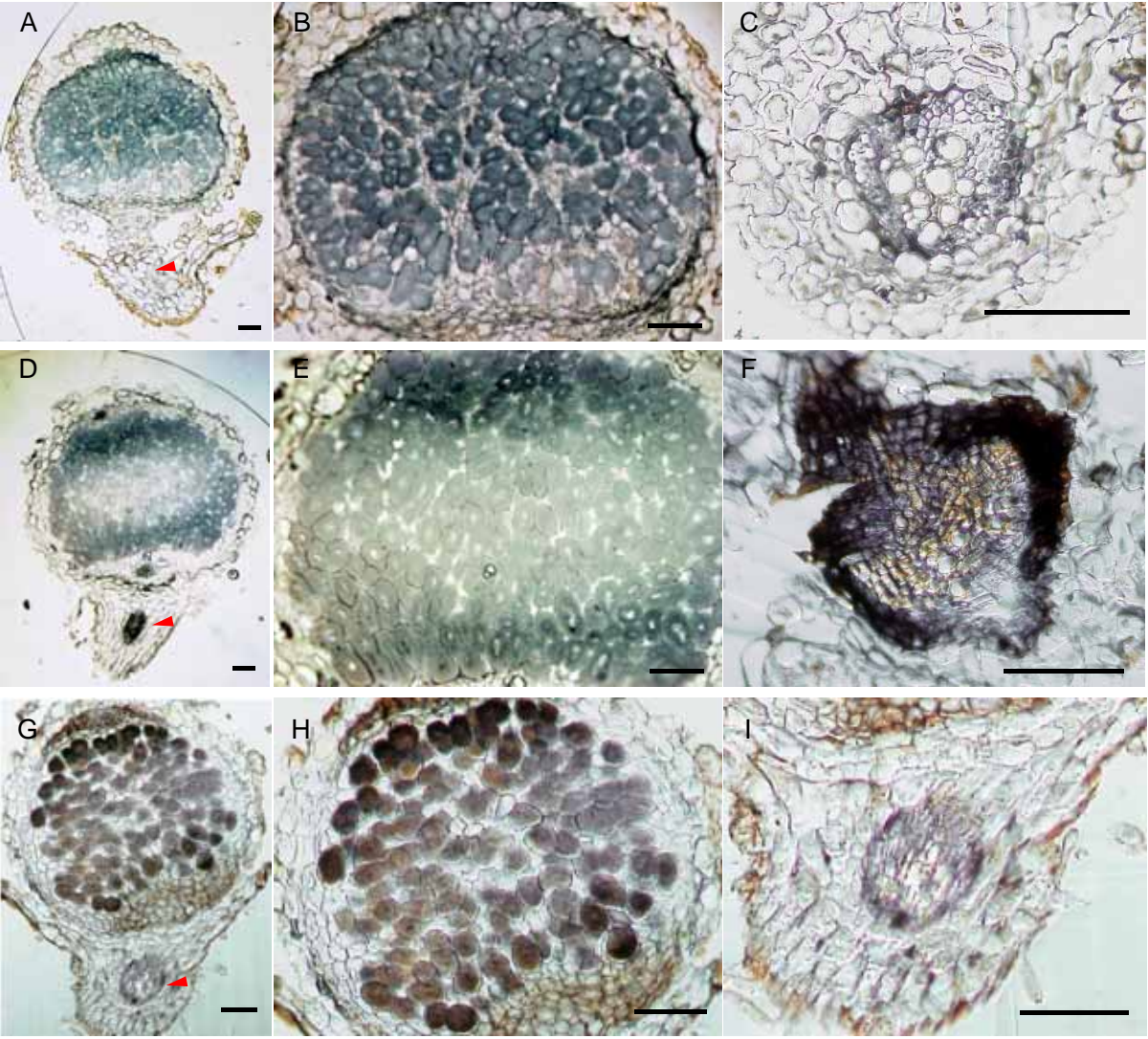
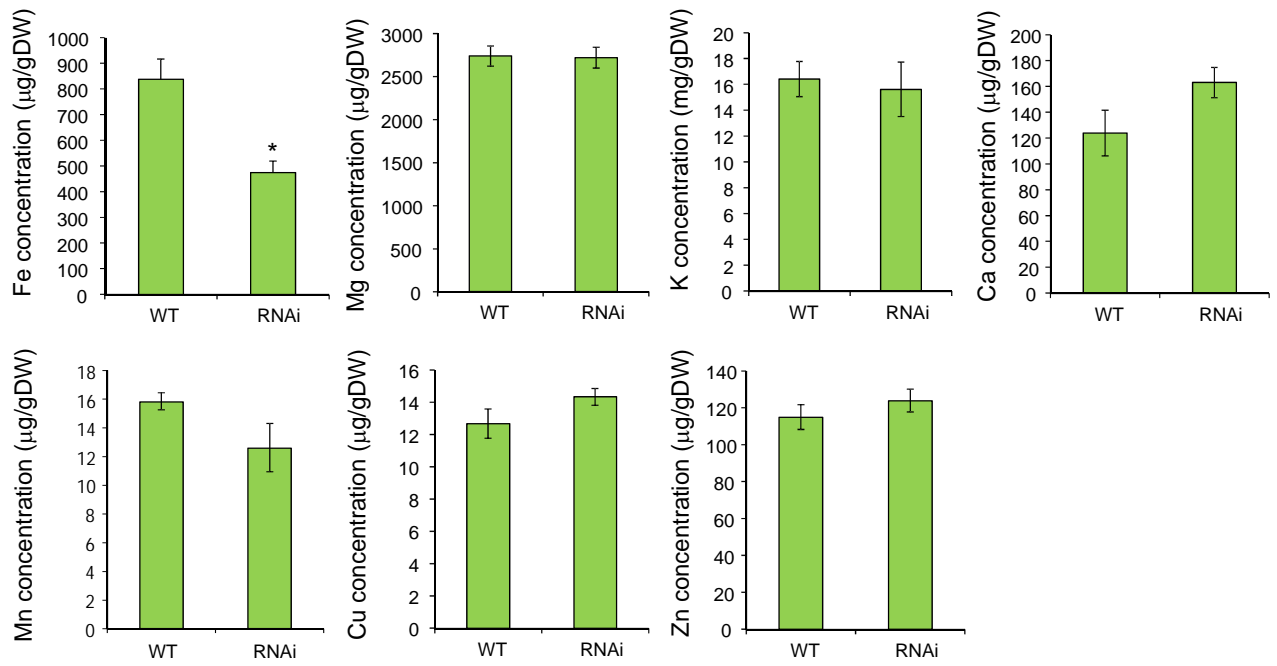
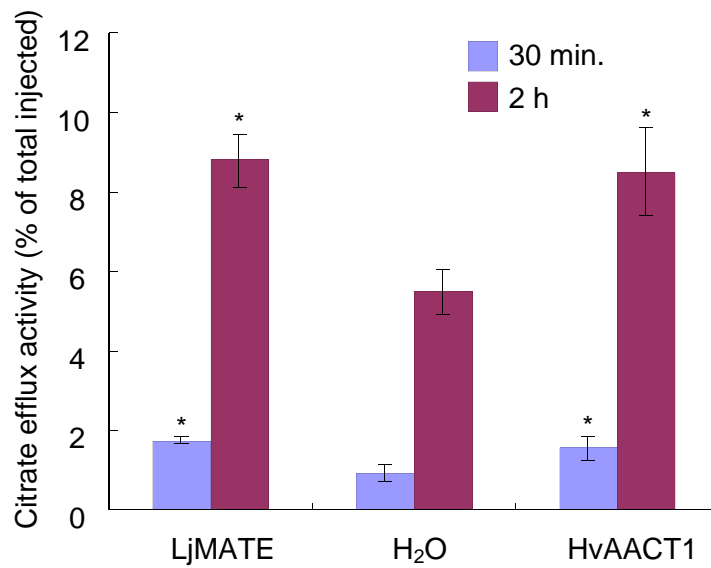


Fig. 8

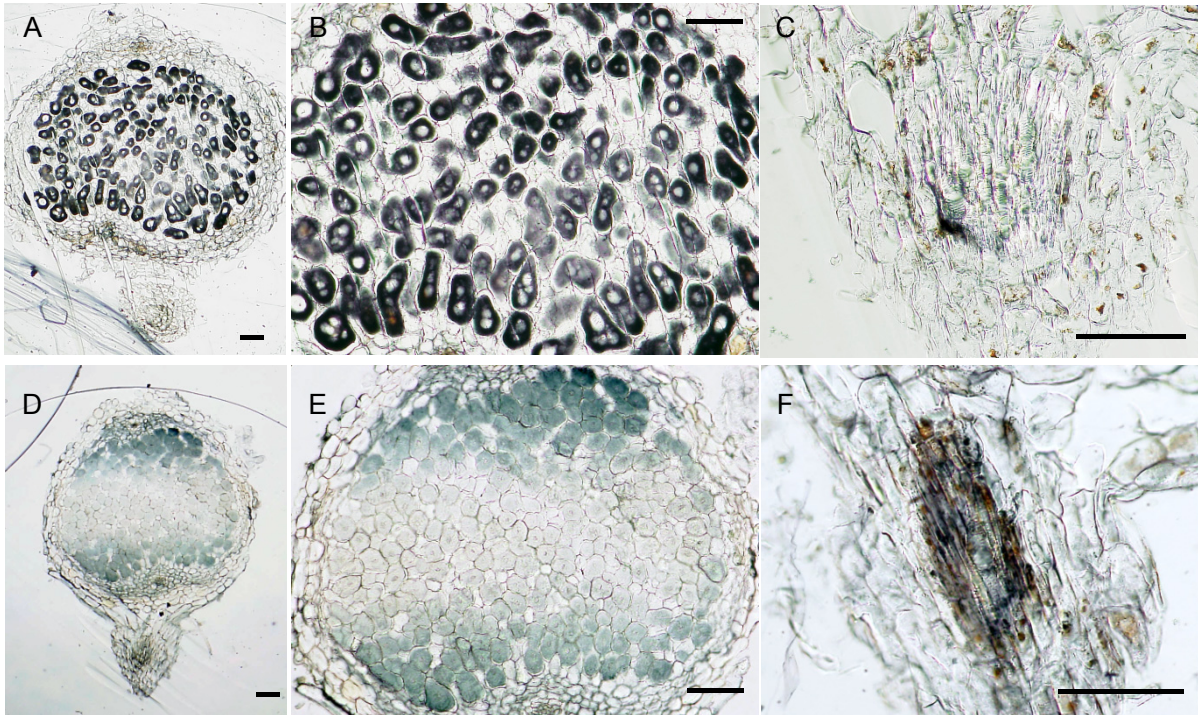


## Supplementary Fig. S1



**Citrate transport activity of LjMATE1 and HvAACT1 measured in *Xenopus* oocytes.** The oocytes were injected with <sup>14</sup>C-labeled citrate, and their release from the oocytes was determined 30 min and 2 h after injection. LjMATE1 showed same efflux activity for citrate with HvAACT1. Values represent mean  $\pm$  SD (n = 3-4). \* $p$ <0.01 compared to control (H<sub>2</sub>O) by Tukey-Kramer test.

## Supplementary Fig. S2



**Typical Fe localization inside the nodules of control (A-C) and hairy root RNAi (D-F).** Perls staining was enhanced by DAB and  $H_2O_2$ . (A, B) High Fe accumulation was observed in infected cells in control nodule. (C) Root vascular bundle was not stained in control. (D, E) Fe accumulation in infection zone was reduced in RNAi, especially in the central region. (F) Abnormality in the Fe accumulation was also detected in root vascular bundle. Scale bars = 100  $\mu$ m.